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Role of AP-1 in the Coordinate Induction of Rat Glutamate-cysteine Ligase and Glutathione Synthetase by *tert*-Butylhydroquinone*

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GSH synthesis occurs via two enzymatic steps catalyzed by glutamate-cysteine ligase (GCL, made up of two subunits) and GSH synthetase (GS). Recently, we described coordinate induction of GCL subunits and GS. To study GS transcriptional regulation, we have cloned and characterized a 2.2-kb 5′-flanking region of the rat GS (GenBank™ accession number AF333982). One transcriptional start site is located at 51 nucleotides upstream of the translational start site. The rat GS promoter drove efficiently luciferase expression in H4IIE cells. Sequential deletion analysis revealed DNA regions that are involved in positive and negative regulation. One repressor identified was NF1. *tert*-Butylhydroquinone (TBH) exerted a dose- and time-dependent increase in the mRNA level and promoter activity of both GCL subunits and GS. TBH increased protein binding to several regions of the GS promoter, c-*jun* expression, and activator protein 1 (AP-1) binding activity to several of the putative AP-1-binding sites of the GS promoter. Blocking AP-1 binding with dominant-negative c-*jun* led to decreased basal expression and significantly blocked the TBH-induced increase in promoter activity and mRNA level of all three genes. In conclusion, AP-1 is required for basal expression of GCL and GS; while NF1 serves as a repressor of GS, increased AP-1 transactivation is the predominant mechanism for coordinate induction of GCL and GS expression by TBH.

GSH is the main non-protein thiol in mammalian cells that participates in many critical cellular functions including antioxidant defense and cell growth (1–3). The synthesis of GSH from its constituent amino acids involves two ATP-requiring enzymatic steps: the formation of γ-glutamylcysteine from glutamate and cysteine, and formation of GSH from γ-glutamylcysteine and glycine. The first step of GSH biosynthesis is generally regarded as rate-limiting and catalyzed by glutamate-cysteine ligase (GCL,[1] also known as γ-glutamylcysteine synthetase), whereas the second step is catalyzed by GSH synthetase (GS) (1). The GCL enzyme is composed of a catalytic (GCLC, M_r ~73,000) and a modifier (GCLM, M_r ~30,000) subunit that are encoded by different genes and dissociate under reducing conditions (4–6). The catalytic subunit exhibits all of the catalytic activity of the isolated enzyme as well as feedback inhibition by GSH (6). The modifier subunit is enzymatically inactive but plays an important regulatory function by lowering the K_m values of GCL for glutamate and raising the K_i value for GSH (5, 7). Because GCL is a major determinant of the overall GSH synthesis capacity, regulation of GCL subunits has been a topic of extensive research (1). Changes in GCL activity can result from regulation at multiple levels affecting only the catalytic or modifier subunit or both. Both human and rat GCL promoters have been cloned (8–12). Antioxidant response element (ARE, also known as electrophile-response element) and activator protein 1 (AP-1) are two *cis*-acting elements present in the promoter of both human GCL subunits that have been implicated in their transcriptional regulation by oxidants and β-naphthoflavone (1, 8–10). In contrast, the cloned 5′-flanking regions of the rat GCL subunits do not contain AREs but contain both AP-1 and nuclear factor κB (NFκB) consensus binding sites (11, 12).

Although much is known about GCL regulation, little attention has been paid to GS. The gene encoding GS was cloned in 1995 (13), but its transcriptional regulation is poorly understood. By using treatments that are known to influence the expression of hepatic GCL subunits, we found that treatments such as *tert*-butylhydroquinone (TBH), diethyl maleate, and buthionine sulfoximine, which increase the expression of both subunits in rat hepatocytes, also increased the expression of GS (14). Increased GS expression further enhanced the capacity of the cell to synthesize GSH. In order to better understand transcriptional regulation of GS, we have cloned and characterized a 2.2-kb 5′-flanking region of the rat GS. In this report, we also examined the mechanism of coordinate induction of rat GCL subunits and GS by TBH.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and fetal bovine serum were obtained from Invitrogen. The Luciferase Assay System and the β-Galactosidase

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF333982.

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[1] The abbreviations used are: GCL, glutamate-cysteine ligase; AP-1, activator protein 1; ARE, antioxidant response element; EMSA, electrophoretic mobility shift assay; GCLC, GCL-catalytic subunit; GCLM, GCL-modifier subunit; GS, GSH synthetase; MZF1, myeloid zinc finger 1; NF1, nuclear factor 1; NFκB, nuclear factor kappa B; Nrf2, nuclear factor-erythroid 2 related factor 2; TBH, *tert*-butylhydroquinone; ANOVA, analysis of variance.

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                                        C/EBP                                                NF1
-2187 AAATATCTGA AATTAGAGTG ATGTGGCTTT  CCAGAACTAA  ACTAACTTGT CTTCCAAGAT TCCTGCCATC
            TCF11          AP1                  TCF11                AP1
-2117 CACCTGGCCT GCTGACCTCA GTGACAGATA  GCCCAGGAGA  TGCTGACCCT TCTCCTGACA TTCTTCTATC

-2047 AGAATCCCCT AGTTAGTCCT AGTCCTGGCA  ATCTTGAGTG  CTTCTTGCTA CCTTCGCTCC CTTCTCCCTT
      cdxA                                                   AP1          HNF3b
-1977 TTTCTAGTCT GTAAGAGAGC AGTAGGCCCC  TCTGGTGGAC  TCTTGACCTA GAGGCATGGA TATTTTCTCA
                                                                 AP1
-1907 GGTTAAACCT GCTTTGTTGT TGAAGTTGCT  TCTTCGTCTT  TTCCTGTCCT GGACCTGTCA GGGGAATGAA
                                      TCF11                                      IX2
-1837 CAAGGTGGGT ACTGGCCATG GTGATGGAAT  TCATGTTCAG  AAGGTACAAA CGACAAAGAG AAGTAATCCC
                                      TCF11                AP1
-1767 AGGCACTAAT ATCTGGTCTG AAGGAGAATC  ATTCACGGGA  CAGACTGACA GGGGTGGTGG TGGTGAACAT
            AP1          AP4       TCF11
-1697 CTGAGTGACA ACGAGCCAGC TGTGTCAGGA  TTTAGAAGTA  AATTATCCAC GGAGAGGAGC AGAAAAGACA
                                      MZF1                 TCF11                AP1
-1627 AAGCTTTGCT GGACGGCATG TTCTTCCTCC  CCTCTCTTGT  CACTTTGCCT CTTTTGTCTC TTGTCACTTT
                                  c-Myb                                  Nrf-2
-1557 GGACAAGTTA GGCAACCTCT CAGCCTCAGT  TGCCATGCCT  ACAAGATGGA AAGGAAGCCA CTTCAGCTGC
                            c-Myb                  NF-κB
-1487 CACAATGTGG AGACCCAACC GAAGGGCTGT  CAACATCTGG  GGTCCTCCCC AAATGTCACC CACAATCACC
            c-Myb                      HSF2
-1417 AGATAGACCA CTGTTGGTCC ATCTTAAAAG  AAGAGTCTGT  ACAGAGCCCT GGGCCTTGGT GCTTTTATTT
      Oct-1          NHF-3b                                                  c-Myb
-1347 TTATTATAAT TTCATCTTTG TTTGTTTTGG  TTTTAAGACA  AGGTCTAACC AACTCTGGCT GGCCTGGAAC
                                                                          AP2
-1277 CGGCTATGTA AACCAGGCTG GTCTAGAACT  CACTGAGATC  CTCCTGCCTC TGCCTCCCAA GGGCTGGGAT
                                  C/EBP-b                          MEF-2
-1207 TAAAGGAACA CGCCAGCACT CCCTGCTTAC  TGTAATTTCT  TTAAGTACTA GAAATAGCTA CCTTTGTCTC
                  AP1              AP1          AP4                          SRY
-1137 TGGTTAATAC AGTGACTTTT GTCTGTCACT  CAAAGCAGCT  GCTTCCTACC TGGTCTGGTA CAGAAACAAG
                  AP1                                          NF1
-1067 AAAAAGGTAA CTGACAGCAA ACATTTTTCT  GGTAATAACC  AGACCACCCA TCTGCCAACT CTGTCCCGTT
                        AP4
 -997 CATACGAGAC ATCTGGACGG CTGTCCTTCC  TACACTGGAG  AGATGTGCCT GCTTTATCTA GAGAGTTAGA
                                       AP1      AP1                              TCF11
 -927 AGGATTCCGG TCCCTGGGTT AGGCCCCTGA  TTTACAGTCA  GTAGAAATTC TCCTTCTAAC TCCCTCTTCA
       AP1          AP1          TCF11                                  NF1
 -857 ATTTGACACC CCTAAAATGT CACCCCTACC  CCCCTTTTTT  AGCTACACAC CCTGGCTTGG TGCCTCACCA
            AP4
 -787 ACTTACAGCT AACTCTACAC GTTTTCCTGT  ACCTAACATA  GAATGGTGTC TTTTCAAATC TCCAGCTTCA
                                       Sp1
 -717 AGATTTCTTC CTGGGGCCAG CTCTTAACAG  CCTGCCCCAC  ACTCTAGAGC CCACCTTTTA TTTACACCGA
                           AP1                          C/EBP,  AP1                TCF11
 -647 ACCGACTAGG AGTTTGGTAT CTTAGTCATA  ATTCCTTGGA  GTTTGGTCAG ATACATTCA TTCATTTAAG
                                  Brn-2                                  SRY
 -577 GAAAATTTTA TCAGGTACCT CAGATTTTGA  ATGAACCTCT  CACACTCTAG GGAAAAGAAA GTTAAATTAC
            TCF11                      TATA                 TCF11                TATA
 -507 TGTAGGTTAC CAGAGGATGA GGGGTTCTGT  ATAGAGGGAA  GAGGATCATG AACTTGCAAA TTTATAAACA
                                                             NF1
 -437 GCCTCGCGGG GCTGAAGAGA GGCCGGCTAA  GAGAACTTTT  GCTCTTGCCA AGGACCCAGG TTCGGTTCCT
                                 AP1          TCF11          AP1      TCF11
 -367 AGGTGATCCG TCGCTTTCTT CTTTGAGTGT  CACCATTTCA  CAGAATGTCA CAAGCTCTTA CTCTCTGAAA
            HNF-3b                 TATA
 -297 ACTAAGCTAA CAGGTAGCAT TCCGCTGTTA  TAGCCACACC  CAAGGCCTCT TCCCTAGAGT AACGACCTAT
                  AP1          TCF11          AP1          Nrf-2          TATA
 -227 CCCTTAAAGT CCAAGTCACT TCTCACCTTT  TCACGCAATG  ACCCTTCCCG GCTGCCGTCC AGTTCTATAG
                        MZF1
 -157 ACGCGGGGCT CCTTCCCCAC CCTCTTTTTT  TTTTTTTTAG  CCTAACAGGG CAGTCTGCGC AGGCGCACTG
      AP1           Sp1
  -87 GGTCAGACCG AAGCCCTGCC CCCTGAGCCT  GGGTAACGGC  GTGTGGAGTT TGAGCCTGGC GAGCAGCTGG
                  Translational start site
  -17 ACAACGAGCG AGTTGGGATG
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FIG. 1. **Nucleotide sequence of the 5′-flanking region of the rat GS gene.** Sequence is *numbered* relative to the translational start site. The putative regulatory elements are indicated in *boldface letters above* the *underlined* sequences.

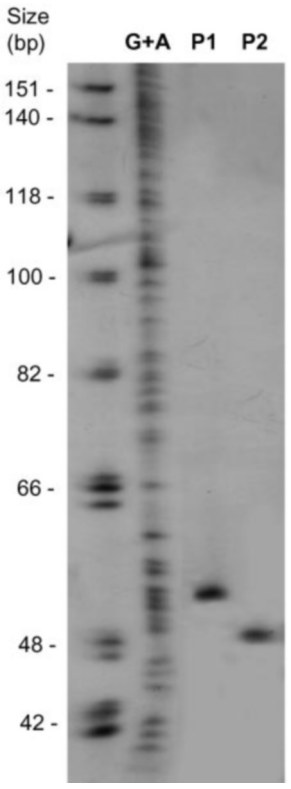

FIG. 2. **Determination of the transcriptional start site of the rat GS gene by primer extension analysis.** Two ^{32}P-end-labeled primers complementary to −24 to +2 (primer 1 or P1) and −27 to −2 (primer 2 or P2) of the rat GS cDNA were annealed to poly(A$^+$) RNA from H4IIE cells or yeast tRNA (primer 2 was used) and extended with Vent DNA polymerase as described under "Experimental Procedures." Size markers correspond to ØX174 digested with *Hin*fI. *Lane G+A* represents a Maxam-Gilbert sequencing reaction in the same fragment.

Enzyme Assay System were obtained from Promega (Madison, WI). All restriction enzymes were obtained from either Promega or Invitrogen. [^{32}P]dCTP and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased PerkinElmer Life Sciences. Total RNA isolation kit was obtained from Invitrogen. All other reagents were of analytical grade and were obtained from commercial sources.

Cloning of the 5′-Flanking Region of the Rat GS Gene—An oligonucleotide probe corresponding to −38 to +2 of the rat GS cDNA (13) was used to screen the rat genomic library EMBL 3 (CLONTECH, Palo Alto, CA). Ten positive plaques were selected; DNA was isolated and digested with *Eco*RI. The insert fragment was subcloned into pGL-3 enhancer vector (Promega) and sequenced in both directions using the automated ABI Prism dRhodamine Terminator Cycle Sequencer performed by the Sequencing Core Facility, Norris Cancer Center, Keck School of Medicine, University of Southern California. The initial primers were universal primers for the pGL-3 enhancer vector, and all subsequent primers were nested primers designed using the available sequence information and the MacVector software program. The nucleotide sequence was verified by multiple bi-directional sequencing reactions. Sequences were aligned, and a consensus sequence was generated using the ASSEMBLIGN software program. A 2.19-kb 5′-flanking region of the rat GS was cloned into the *Sma*I site of promoterless pGL-3 enhancer vector creating the recombinant plasmid −2187/+2 GS-LUC.

Primer Extension Analysis—Primer extension analysis was done as described (11). Two antisense oligonucleotide primers complementary to −24 to +2 and −27 to −2 nucleotides relative to the translational start site of the rat GS (13) were end-labeled with [γ-^{32}P]ATP using T_4 polynucleotide kinase. Two and a half μg of poly(A$^+$) RNA from H4IIE cells isolated as described (15) was annealed to 10^6 cpm of the primers and extended with 4 units of Vent DNA polymerase (New England Biolabs, Inc.). The primer extended products were analyzed on 7 M urea, 6% polyacrylamide gels.

Construction of 5′-Deletion Constructs—The 2.19-kb fragment in the sense orientation upstream of the luciferase coding sequence of the pGL-3 enhancer vector (Promega) is the construct that contains the longest 5′-flanking sequence (−2187 to +2) employed in the transfection assay. To prepare 5′-deletion constructs, this plasmid was subjected to digestion with additional restriction enzymes to generate a series of deletion mutants. The enhancer/reporter transgene −1808/+2 GS-LUC was created by cloning an *Eco*RI fragment. −1164/+2 GS-LUC was created by using the Erase-a-Base System kit (Promega), which uses an exonuclease III that digests only 5′-overhangs or blunt-ended sites. The resulting DNA fragment containing the luciferase expression vector was blunt-ended by Klenow (Invitrogen) and self-ligated by T4 DNA ligase. −561/+2 GS-LUC was created by cloning a *Kpn*I fragment, and −252/+2 GS-LUC was created by cloning a *Stu*I fragment.

Recombinant Plasmids and Adenoviral Vectors—Rat GCLC and GCLM promoter-luciferase constructs were previously described (11, 12). Recombinant, replication-defective adenovirus expressing dominant-negative c-*jun*, TAM67, was kindly provided by Dr. David Brenner (16). TAM67 is a truncation mutant of c-*jun* that lacks the transactivation domain, whereas the DNA binding domain remains intact, permitting dimerization with other AP-1 family members and inhibiting AP-1-mediated transcription (16).

Infection of H4IIE Cells with Adenovirus Encoding TAM67—Recombinant adenoviruses encoding TAM67 or empty vector were amplified in 293 cells. H4IIE cells grown according to instructions provided (American Type Culture Collection number CRL-1548) were infected with unpurified recombinant adenovirus encoding for TAM67 or empty vector at multiplicity of 20 plaque-forming units/cell for 24 h. In preliminary experiments, this condition yielded maximum expression of TAM67 as determined by Northern blot analysis (not shown). After 24 h of infection, the viruses were removed and replaced with fresh medium for transfection analysis as described below.

Analysis of Promoter Constructs in Cell Culture—Relative transcriptional activity of the GS promoter fragments was studied using H4IIE cells transiently transfected with GS promoter-luciferase constructs for 7 h using the Superfect Transfection Reagent (Qiagen, Valencia, CA) as we described previously (11, 12). To study the effect of nuclear factor 1 (NF1) binding on GS promoter activity, the NF1 site at −1025 (NF1-1) was mutated from ACCAGACCACCCATCTGCCAACTCTGT to ACCAGACCACCCATCT**TAA**AACTCTGT (NF1m1), and the NF1 site at

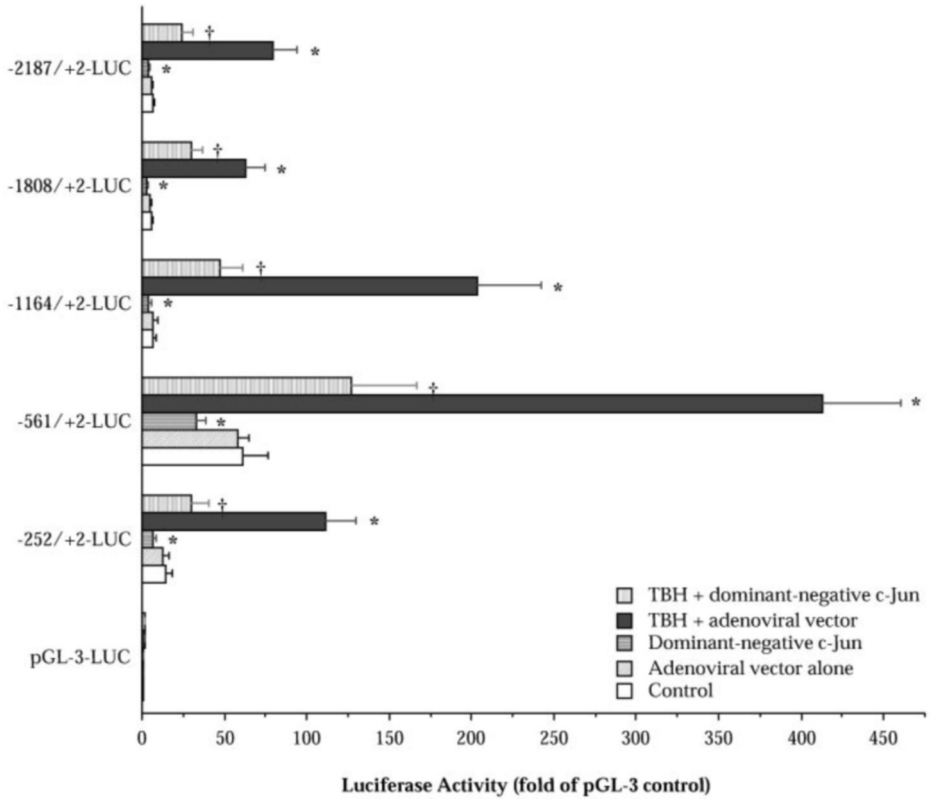

FIG. 3. **Transient transfection analysis of the rat GS promoter-luciferase constructs in H4IIE cells.** Progressive 5′-deletions of the GS promoter extending from −2187 to +2 bp were generated and fused to the promoterless luciferase pGL-3 enhancer vector as described under "Experimental Procedures." Numbering is defined relative to the translational start site. In some experiments, cells were previously infected with adenoviral vectors encoding dominant-negative c-*jun* or adenoviral vectors alone prior to transfection with GS promoter-luciferase constructs. Cells were then treated with vehicle control (Me_2SO) or TBH (60 μM for 4 h) prior to cell harvest. Results represent mean ± S.E. from three to six independent experiments performed in triplicate. Data are expressed as relative luciferase activity to that of pGL-3 enhancer vector control, which is assigned a value of 1.0. *, $p < 0.05$ *versus* respective control or adenoviral vector alone; †, $p < 0.05$ *versus* TBH + adenoviral vector treated (ANOVA followed by Fisher's test).

−808 (NF1-2) was mutated from TACACACCCTGGCTTGGTGCCTCACCA to TACACACCCTGGCTTGGT**TAA**TCACCA (NF1m2), where the binding sites are underlined and the mutated sequence is shown in bold. Mutation was performed by PCR and confirmed by restriction enzyme digestion and sequencing. H4IIE cells were transfected with −1164/+2 GS-LUC that contained wild type, NF1m1 (first NF1 site is mutated) or NF1m1m2 (both NF1 sites are mutated), and the effect of the mutation was assessed by measuring luciferase activity.

The effect of TBH on GS promoter activity was examined by measuring luciferase activity driven by GS promoter luciferase gene constructs in transfected H4IIE cells treated with TBH (60 μM) during the last 4 h of the transfection. To study the effect of blocking AP-1 activity, H4IIE cells infected previously with TAM67 or adenoviral vector alone were transfected with GS promoter constructs and treated with TBH (60 μM for 4 h). The same types of studies were also carried out using rat GCLC and GCLM promoter constructs.

Effect of TBH on the Expression of GCL Subunits and GS in H4IIE Cells—H4IIE cells were treated with 0–120 μM TBH for 4 h or 60 μM for 0–12 h. In some experiments, cells had been infected with adenovirus encoding dominant-negative c-*jun* or adenovirus vector alone prior to TBH treatment. At the end of the TBH treatment, total RNA was extracted, and Northern hybridization analysis was performed using specific rat GCLC, GCLM, GS, c-*jun*, and β-actin cDNA probes as described (14). The c-*jun* cDNA probe corresponds to nucleotides 362–882 of the published rat c-*jun* sequence (17) and was obtained by reverse transcription and PCR using a one-step reverse transcriptase-PCR kit (CLONTECH). Results of Northern blot analysis were normalized to β-actin.

DNase I Footprinting Analysis—^{32}P-End-labeled fragments of the 5′-flanking region of rat GS strongly induced by TBH treatment were generated by digestion with restriction endonucleases. DNase I footprinting analysis was performed using double-stranded fragments corresponding to nucleotides −1207 to −997, −938 to −769, −768 to −561, −563 to −252, and −252 to +2 of the rat GS gene. These fragments were cloned into the TA vector (Invitrogen). Singly end-labeled fragments were generated with [γ-^{32}P]ATP using T_4 polynucleotide kinase, and the DNase I footprinting procedure was performed as we described previously (11).

EMSA and Supershift Assay—EMSAs for different regions of the rat GS promoter were done as described previously (11, 12) using 5–15 μg of nuclear protein from H4IIE cells treated with TBH (60 μM for 4 h) or vehicle control (Me_2SO). The probes used included putative AP-1-binding sites (11 nucleotides long) at −1098, −902, −896, −856, −844, −607, −343, −339, −326, −218, −192, and −91 (flanked by 5′-CTTGA and GCCGG-3′ for each probe), Sp1-binding site at −689 (DNA fragment corresponds to −694 to −674), NF1-binding sites at −1025 (NF1-1), and −808 (NF1-2) that contain either wild type or mutated sequence (NF1m1 and NF1m2) as described above.

Supershift analysis was performed with specific anti-c-*jun*, anti-Sp1, and anti-NF1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (11). To assess the effect of dominant-negative c-*jun*, cells were previously infected with TAM67 and subsequently treated with TBH (60 μM for 4 h) and processed for EMSA and supershift analysis.

Statistical Analysis—Data are given as mean ± S.E. Statistical analysis was performed using ANOVA followed by Fisher's test for multiple comparisons. For changes in mRNA levels, ratios of GCL subunits and GS to β-actin densitometric values were compared by ANOVA. Significance was defined by $p < 0.05$.

RESULTS

Cloning and Sequencing of the 5′-Flanking Region of the Rat GS—The sequence of the 2.19-kb product is shown in Fig. 1. Analysis of the transcription factor binding site was done using Transcription Factor Search (www.cbrc.jp/research/db/TFSEARCH.html) and MatInspector version 2.2 (www.gsf.de/cgi-bin/matsearch.pl). The 5′-flanking region of the rat GS contains numerous consensus binding sites for AP-1 and transcription factor 11. It also contains several consensus binding sites for CAAT enhancer-binding protein, myeloid zinc finger 1, NF1, nuclear factor-erythroid 2 related factor 2, hepatocyte-enriched nuclear factor-3b, Sp1, and one binding site for NFκB and heat shock transcription factor 2.

Transcriptional Start Site—Primer extension was used to determine the transcriptional start site. Two antisense oligonucleotide primers complementary to −24 to +2 (primer 1) and −27 to −2 (primer 2) nucleotides relative to the translational start site of the rat GS (13) were annealed to poly(A^+) RNA from H4IIE cells and extended toward the 5′ end of the mRNA by reverse transcription. Fig. 2 shows the primer extension reaction yielded products of ~53 and 49 nucleotides long using primers 1 and 2, respectively. These products were not detected

when the assay was carried out using tRNA (not shown). These results are consistent with the transcriptional start site being located 51 nucleotides upstream of the translational start site.

Functional Analysis of the 5′-Flanking Region of Rat GS—To delineate sequences that drive the expression of the rat GS, five 5′-terminal nested deletion mutants ranging from −2187/+2 to −252/+2 were cloned into the promoterless luciferase reporter gene vector pGL3 enhancer. The promoterless construct pGL3 enhancer served as the background control. Luciferase activity was measured after transient transfection of H4IIE cells with these constructs. Fig. 3 shows that the rat GS promoter was able to drive efficiently luciferase expression in H4IIE cells. The construct −561/+2 produced maximal promoter activity (61-fold increase over pGL-3) whereas the construct −1164/+2 produced about 11% of maximal activity, indicating presence of important elements between −561 and −51 and −1164 and −561 that positively or negatively regulated the promoter activity, respectively. Inclusion of an additional 1 kb upstream had no significant influence on the promoter activity.

Transcription Factor NF1 Mediates Repression of the Rat GS Gene—We next investigated the mechanism of repression in the region between −1164 and −561 of the GS promoter. Of the consensus binding sites present in this region, NF1 is of interest because it has been described to act as a transcriptional repressor (18, 19). To examine the effect of the two NF1-binding sites on the GS promoter, we performed EMSA with supershift analysis using double-stranded probes that span these two NF1 sites that contain either wild type or mutated sequence, and we measured luciferase activity driven by the GS promoter construct −1164/+2-LUC that contains wild type, NF1m1 (first NF1 site is mutated), or NF1m1m2 (both NF1 sites are mutated). Fig. 4 shows that NF1 binds to both of these sites, but especially to site 2, and mutation of the first NF1 site led to a 5-fold increase in luciferase activity, whereas mutation of both NF1 sites led to a 16-fold increase in luciferase activity.

Effect of TBH on GCL, GS, and c-jun Expression in H4IIE Cells—We had shown previously that TBH induced the expression of both GCL subunits and GS in rat hepatocytes (14, 20). By having cloned the promoter region of all three genes, we are poised to investigate the molecular mechanism(s) responsible for the coordinate induction in gene expression. We first established the dose- and time-dependent effect of TBH on the expression of GSH synthetic enzymes in H4IIE cells (Fig. 5, *A* and *B*). Maximum induction was observed with 4 h of treatment of 60 μM TBH, which was used as the treatment protocol for all subsequent studies involving TBH. TBH also induced a transient increase in the mRNA level of c-*jun* (Fig. 5*C*).

Effect of TBH on GCL and GS Promoter Activity in H4IIE Cells—We next examined the effect of TBH treatment on the promoter activity of GCL subunits and GS. Fig. 3 shows that TBH treatment induced markedly the reporter activity driven by the GS promoter, particularly the promoter construct −1164 to +2, where TBH led to a 35-fold increase in activity. Table I summarizes the effect of TBH treatment on the promoter activity of the two GCL subunits. Similar to the effect on GS promoter, TBH also induced markedly the activity of both GCL subunit promoters. Maximum induction was seen with the GCLC construct −707/+2-LUC (about 10-fold over control) and GCLM construct −441/+1-LUC (about 13-fold over control).

DNase I Footprinting Analysis of Rat GS 5′-Flanking Region—To delineate the *cis*-acting elements that may be involved in mediating the effect of TBH on the GS promoter, DNase I footprinting analysis of the 1.2-kb 5′-flanking region of the GS gene was carried out. Fig. 6 shows footprinting results using probes that span the region −1207 to +2. Nuclear protein-dependent DNase I-protected areas are present in regions

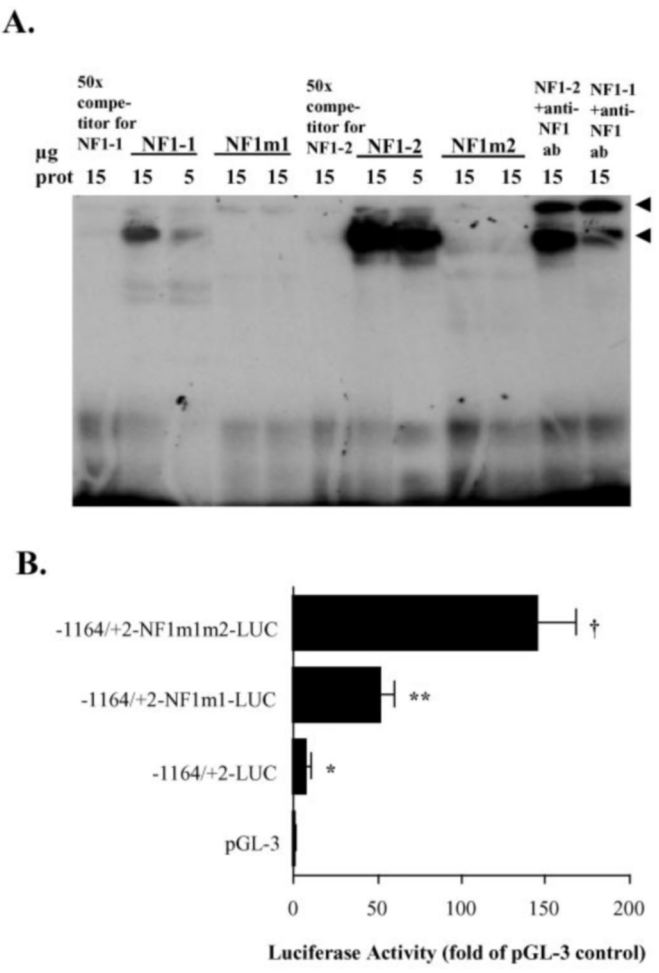

FIG. 4. **Role of NF1 binding on GS promoter activity.** NF1 binding to two sites (NF1-1 at −1025 and NF1-2 at −808) of the GS promoter was assessed by EMSA and supershift (*A*). Nuclear protein extracts (5–15 μg) were obtained from H4IIE cells, and EMSA with supershift was done using probes that span the two NF1 sites that contain either wild type or mutated sequence (NF1m1 and NF1m2) as described under "Experimental Procedures." The *arrowheads* to the *right* point to specific complexes that were competitively blocked when 15 μg of nuclear protein was incubated with radiolabeled probes in the presence of 50× unlabeled specific probes (*lower arrowhead*) and supershifted in the presence of specific antibodies to NF1 (*top arrowhead*). Effect of mutating the NF1-binding sites on GS promoter activity was assessed by measuring luciferase activity in H4IIE cells transfected with −1164/+2 GS-LUC that contained wild type, NF1m1 (first NF1 site is mutated), or NF1m1m2 (both NF1 sites are mutated) (*B*). Results represent mean ± S.E. from six independent experiments performed in triplicate. Data are expressed as relative luciferase activity to that of pGL-3 enhancer vector control, which is assigned a value of 1.0. *, $p < 0.05$ *versus* pGL-3; **, $p < 0.05$ *versus* pGL-3 and −1164/+2-LUC; †, $p < 0.05$ *versus* pGL-3, −1164/+2-LUC, and −1164/+2-NF1m1-LUC (ANOVA followed by Fisher's test).

−1065 to −1036, −901 to −830, −707 to −679, −354 to −314, and −104 to −68. Except for the region −707 to −679, the other regions all contain multiple AP-1-binding sites.

EMSA and Supershift Analysis—Because AP-1 is known to be induced by TBH (21) and there are 12 AP-1-binding sites in the 1.08-kb 5′-flanking region of the GS gene, we next examined which of these may be affected by TBH treatment. Fig. 7 shows increased AP-1 binding to six of the potential AP-1 sites (−1058, −896, −844, −343, −326, and −91) in response to TBH treatment. This was confirmed by supershift analysis using anti-c-*jun* antibodies (Fig. 7). However, the other six potential AP-1-binding sites (−902, −856, −607, −339, −218, and −192) exhibited no change in AP-1 binding in response to TBH treatment (data not shown).

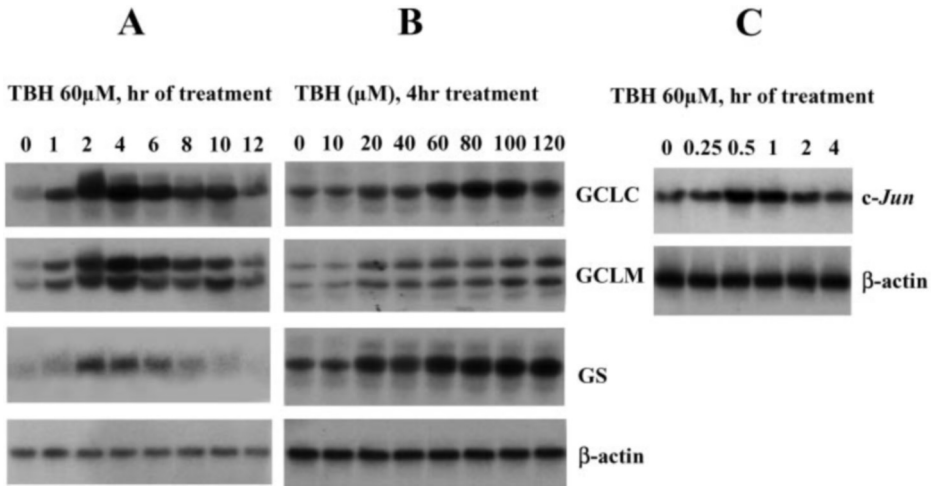

FIG. 5. **Effect of TBH on GCL subunits and GS and c-*jun* expression in H4IIE cells.** RNA (25 μg/lane) samples from H4IIE cells treated with 60 μM TBH for 0–12 h (*A*), 0–120 μM for 4 h (*B*), or 60 μM for 0–4 h (*C*) were analyzed by Northern blot analysis with a ^{32}P-labeled GCLC cDNA or c-*jun* probe as described under "Experimental Procedures." The same membrane was then sequentially rehybridized with ^{32}P-labeled GCLM, GS, and β-actin cDNA probes (*A* and *B*) or only β-actin cDNA probe (*C*). Representative Northern blots are shown.

TABLE I

Effects of TBH and dominant-negative c-Jun on GCLC and GCLM promoter activities

Results represent mean ± S.E. from 3 to 6 separate experiments expressed as fold of pGL-3 enhancer vector. H4IIE cells were transfected with GCLC or GCLM promoter-luciferase constructs and treated with 60 μM TBH for the last 4 h of the transfection as described under "Experimental Procedures." To examine the effect of dominant-negative c-*Jun* (DN c-*Jun*), H4IIE cells were previously infected with adenoviral vectors encoding DN *c-Jun* or adenoviral vectors alone prior to transfection with −707/+2 GCLC-LUC or −441/+1 GCLM-LUC constructs. Cells were then treated with vehicle control (Me_2SO) or TBH (60 μM) for 4 h). Adenoviral vector alone and Me_2SO had no effect on the luciferase activity.

Promoter constructs	Control	TBH	DN c-*Jun*	DN c-*Jun* + TBH
GCLC				
pGL-3-LUC	1.0 ± 0	0.9 ± 0.03		
−113/+2-LUC	1.7 ± 0.1	1.9 ± 0.1		
−597/+2-LUC	87.4 ± 0.9	387.6 ± 26.0[a]		
−707/+2-LUC	48.7 ± 6.3	474.8 ± 10.5[a]	24.1 ± 4.5[a]	116.7 ± 11.6[b]
−1110/+2-LUC	75.2 ± 7.0	427.6 ± 36.2[a]		
−1760/+2-LUC	62.1 ± 7.5	418.0 ± 21.6[a]		
GCLM				
pGL-3-LUC	1.0 ± 0	1.2 ± 0.1		
−154/+1-LUC	2.6 ± 0.2	40.4 ± 6.7[a]		
−441/+1-LUC	18.6 ± 3.2	247.1 ± 26.5[a]	8.6 ± 0.8[a]	44.8 ± 2.9[b]
−649/+1-LUC	86.0 ± 9.7	231.9 ± 31.9[a]		
−850/+1-LUC	57.2 ± 3.6	148.5 ± 15.4[a]		
−1251/+1-LUC	8.6 ± 1.9	20.6 ± 4.2[a]		
−1850/+1-LUC	8.7 ± 2.2	18.9 ± 4.9[a]		

[a] $p < 0.05$ versus respective controls.
[b] $p < 0.05$ *versus* TBH treatment by ANOVA.

Although the region −707 to −679 of the GS promoter does not contain AP-1 element, it contains an Sp1 element (−689 to −680). We next investigated whether TBH treatment also induced Sp1 binding. Fig. 8 shows that there is also a striking increase in Sp1 binding to this Sp1 site in response to TBH.

Role of AP-1 in Basal and TBH-induced Increase in the Expression of GSH Synthetic Enzymes—To evaluate the importance of AP-1 in both the basal expression and in the TBH-mediated increase in expression of GSH synthetic enzymes, H4IIE cells were infected with dominant-negative c-*jun,* and the effect of blocking AP-1 activity on TBH-mediated changes was examined. Fig. 9 shows that the basal expression of both GCL subunits and GS fell when AP-1 activity was blocked (both GCLM and GS decreased by more than 50%; GCLC fell by 30%). The TBH-mediated increase in the mRNA level of all three genes was also significantly blocked.

Dominant-negative c-*jun* also blocked significantly the TBH-mediated increase in AP-1 binding to the GS promoter (Fig. 7), and reporter activity driven by GCLC, GCLM, and GS promoters (Fig. 3 and Table I). Note that the basal promoter activity was reduced by 40–50% in all of the promoter constructs (Fig. 3 and Table I).

In addition to blocking AP-1 binding to the GS promoter, dominant-negative c-*jun* also reduced significantly Sp1 binding to the Sp1 site at −689 to −680 of the GS promoter under basal conditions and in response to TBH treatment (Fig. 8). Note that c-*jun* does not bind directly to the Sp1 site as supershift occurred with anti-Sp1 antibodies but not anti-c-*jun* antibodies (Fig. 8).

DISCUSSION

GSH is the most abundant non-protein thiol that is important in numerous cellular processes including antioxidant defense, storage of cysteine, and maintenance of the redox state (1). One of the major determinants of the synthesis of GSH is the activity of GCL. Because of its importance, regulation of GCL has been a topic of extensive research (1). In contrast, little attention has been paid to GS. GS deficiency in humans can result in dramatic metabolic consequences because the accumulated γ-glutamylcysteine is converted to 5-oxoproline which can cause severe metabolic acidosis, hemolytic anemia, and central nervous system damage (13, 22). Mutation inactivation of GS has been described (22). Recently two reports have shown a selective fall in GS activity which resulted in lowered GSH level (23, 24). If GCL is rate-limiting, how can a change in GS activity influence the steady state GSH level? Although the specific activity of GS is normally 2–4 times that of GCL activity in normal liver (25), this may not be the case in other tissues. In fact, in normal human skeletal muscle, the specific activity of GS was only 36% higher than that of GCL (24).

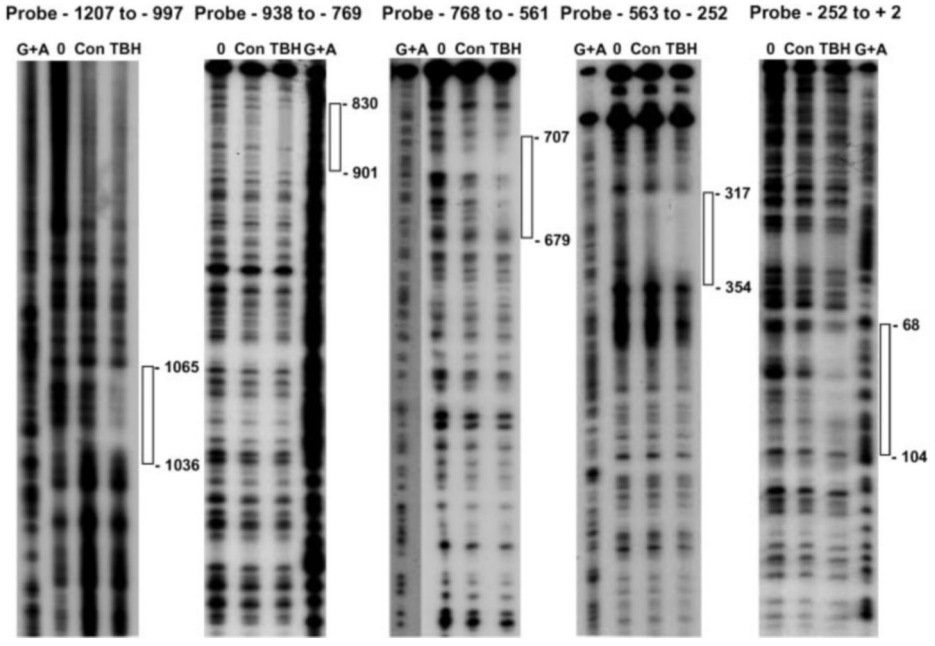

FIG. 6. **Effect of TBH treatment on DNase I footprinting analysis of the −1207 to −997, −938 to −769, −768 to −561, −563 to −252, and −252 to +2 regions of the rat GS promoter.** DNA fragment containing −1207 to −997 (*lower strand*), −938 to −769 (*upper strand*), −768 to −561 (*lower strand*), −563 to −252 (*upper strand*), and −252 to +2 (*upper strand*) regions of the rat GS promoter were end-labeled and digested with DNase I in the absence (*0*) or presence of 10 μg of nuclear protein extracts from H4IIE cells treated with vehicle control (*Con*) (Me_2SO) or TBH (60 μM for 4 h). Positions of the protected regions are indicated at the *right* of the figures. *Lanes G+A* represent a Maxam-Gilbert sequencing reaction in the same fragments.

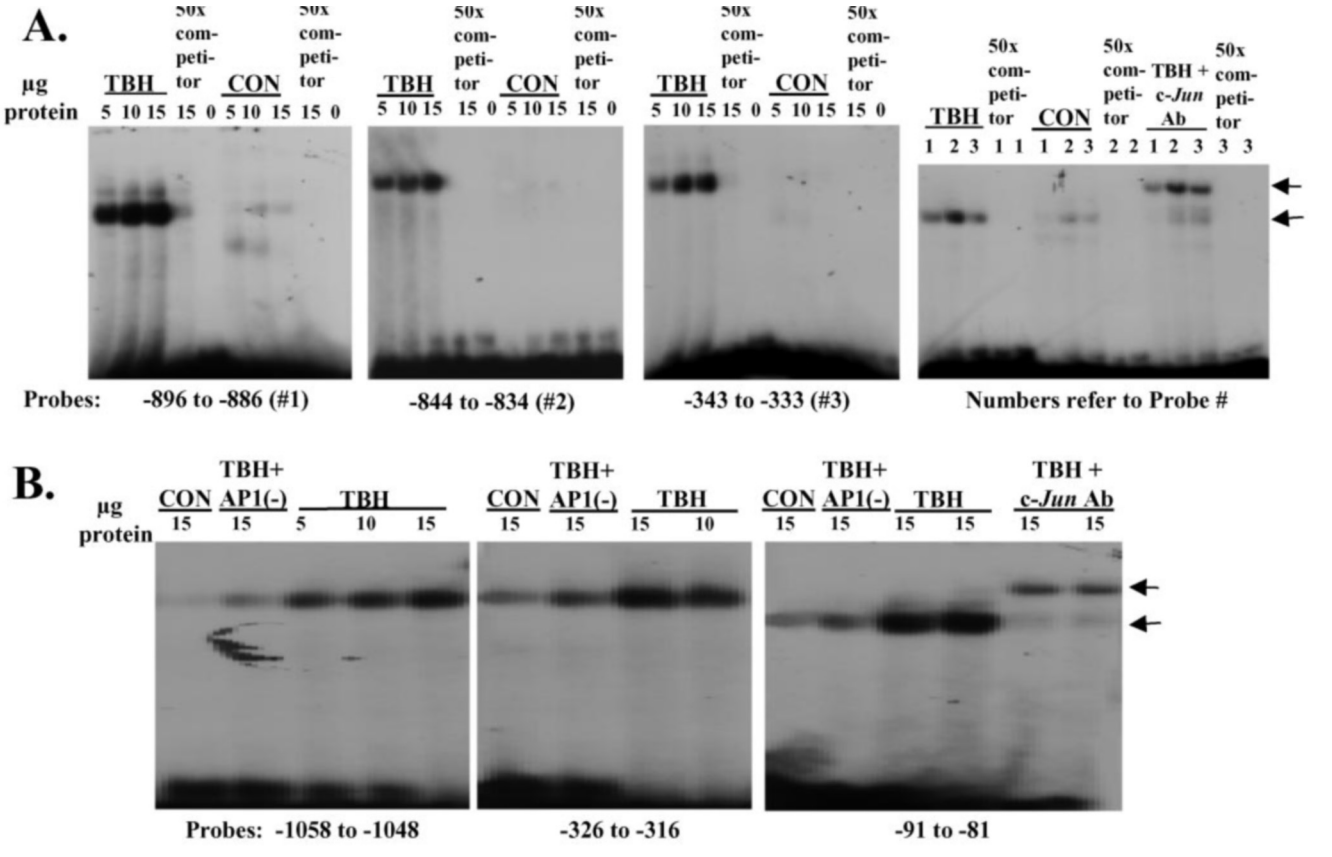

FIG. 7. **Effect of TBH and dominant-negative c-*jun* (AP1(−)) on electrophoretic mobility shift and supershift assays for different AP-1-binding sites of the rat GS promoter.** Nuclear protein extracts (5–15 μg) were obtained from H4IIE cells treated with vehicle control (*CON*) (Me_2SO) or TBH (60 μM for 4 h), and EMSA with supershift was done as described under "Experimental Procedures" using probes that span different AP-1-binding sites of the rat GS promoter (*A*). *B*, H4IIE cells were infected with adenoviral vectors encoding dominant-negative c-*jun* (AP1(−)) or adenoviral vector alone and subsequently treated with TBH. Note that TBH treatment led to increased AP-1 binding that was inhibited significantly if cells were treated previously with dominant-negative c-*jun*. Similar findings were obtained with probes −896 to −886, −844 to −834, and −343 to −333 (not shown). The *arrows* to the *right* point to specific complexes that were competitively blocked when 15 μg of nuclear protein from TBH-treated cells was incubated with radiolabeled probes in the presence of 50× unlabeled specific probes (*lower arrow*) and supershifted in the presence of specific antibodies to c-*jun* (*top arrow*).

Surgical trauma selectively reduced GS activity, which became rate-limiting (24). Collectively these results suggest regulation of GS has been overlooked and may be just as important in determining the overall GSH synthetic capacity as GCL under certain conditions and in non-hepatic tissues.

By using treatments that are known to influence the hepatic GCL subunit expression, we found treatments that increase the expression of both GCL subunits also increased the expres-

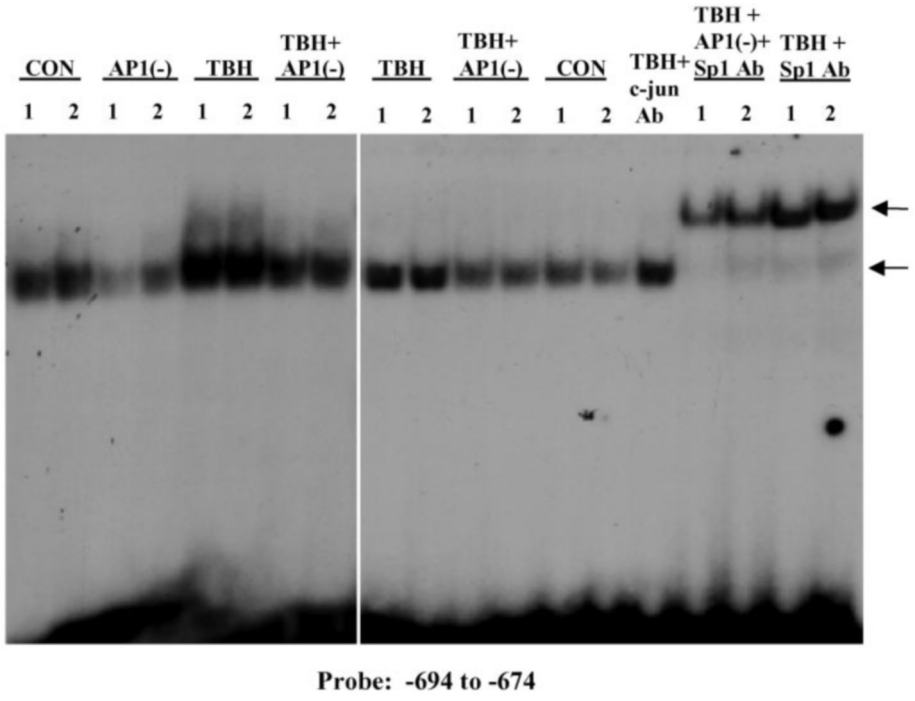

FIG. 8. **Effect of TBH and dominant-negative c-*jun* (AP1(−)) on electrophoretic mobility shift and supershift assays for Sp1-binding site at −689 of the rat GS promoter.** H4IIE cells were infected with adenoviral vectors encoding dominant-negative c-*jun* or adenoviral vectors alone and subsequently treated with TBH (60 μM for 4 h) or vehicle control (*CON*). Nuclear protein extracts (15 μg) were obtained after various treatments, and EMSA was done as described under "Experimental Procedures" using probe −694 to −674 of the GS promoter. The *arrows* are pointing to the TBH-induced increase in Sp1 binding (*lower arrow*) which is confirmed by supershift analysis using specific anti-Sp1 antibodies (*upper arrow*). No supershift occurred in the presence of anti-c-Jun antibodies (*Ab*).

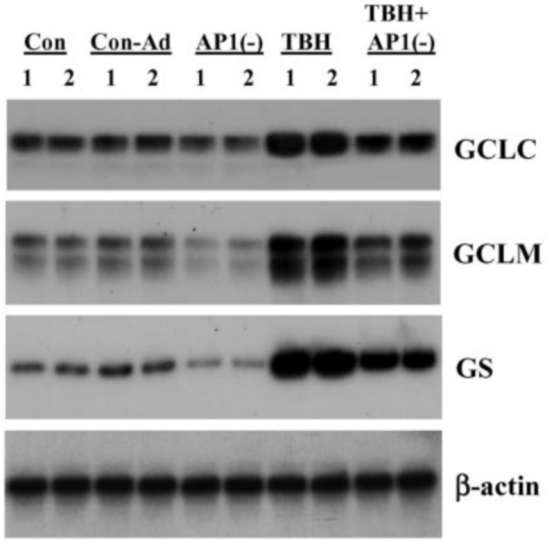

FIG. 9. **Effect of TBH and dominant-negative c-*jun* (AP1(−)) on steady state mRNA levels of GCL subunits and GS.** H4IIE cells were infected with adenoviral vectors encoding dominant-negative c-*jun* or adenoviral (*Ad*) vectors alone and subsequently treated with TBH (60 μM for 4 h) or vehicle control (*Con*). Northern blot analysis was carried out with RNA (25 μg/lane) samples after various treatments using a ^{32}P-labeled GCLC cDNA probe as described under "Experimental Procedures." The same membrane was then sequentially rehybridized with ^{32}P-labeled GCLM, GS, and β-actin cDNA probes. Representative Northern blots are shown.

sion of GS (14). This coordinated induction in GS and GCL led to higher GSH synthetic capacity than induction in GCL alone (14). To better understand GS transcriptional regulation and how coordinated regulation of GSH synthetic enzymes occur, we have cloned and characterized the rat GS promoter and elucidated the molecular mechanism of TBH-induced coordinated increase in the expression of GSH synthetic enzymes.

The sequence of 5′-flanking region of the rat GS contains numerous AP-1 and transcription factor 11-binding sites but no ARE site. This is similar to the rat GCL subunit promoters, which also lack ARE elements (11, 12). Primer extension analysis revealed a single transcriptional start site located 51 nucleotides upstream of the translational start site. Transfection studies showed that the 5′-flanking sequence of the rat GS gene contains a functional promoter that was able to drive luciferase expression in H4IIE cells efficiently. Maximal promoter activity was obtained with the GS promoter construct −561/+2-LUC, denoting the presence of enhancer element(s) in this region. In contrast, the presence of repressor element(s) is suggested between −1164 and −561 as the promoter activity fell almost 9-fold. By using EMSA, supershift, and site-directed mutagenesis analyses, we found NF1 to be a strong repressor of the rat GS promoter. NF1 binds to both sites (−1025 and −808), and when binding is prevented by mutagenesis, the resulting promoter activity surpassed the maximal activity obtained with the −561/+2-LUC construct, suggesting that many of the enhancer elements (*i.e.* the AP-1 sites) in the region between −1164 and −561 were suppressed.

By having cloned the promoter region of both GCL subunits (11, 12) and GS in the rat and having shown that TBH induces comparably the expression of all three genes in rat hepatocytes (14), we next examined the molecular mechanism(s) of this coordinate induction. TBH has been shown by others to induce the nuclear binding activity of AP-1, NFκB, and ARE (21, 26). However, it has also been shown to induce the human GCLM promoter activity independent of ARE or AP-1 (27). Similar to rat hepatocytes, TBH exerted a comparable increase in the mRNA levels of both GCL subunits and GS in the rat hepatoma cell line H4IIE in a dose- and time-dependent manner. TBH also induced strongly the reporter activity driven by all three promoter-luciferase constructs, although with varying magnitude depending on the length of the construct. DNase I footprinting analysis was next used to elucidate possible *cis*-acting elements involved in mediating the effect of TBH. After TBH treatment, there was a dramatic increase in protein binding to several regions of the GS promoter, and all except one is rich in AP-1 sites. EMSA with supershift was then used to see which of the 12 potential AP-1 sites present in the 1.1-kb 5′-flanking region of the GS gene exhibited increased binding after TBH treatment. Consistent with results obtained with DNase I footprinting, increased AP-1 binding activity was confirmed with EMSA and supershift analysis to putative AP-1-binding sites at −1058, −896, −844, −343, −326, and −91. Similarly, no

increase in protein or AP-1 binding activity was detected to putative AP-1-binding sites at −607, −218 or −192 with DNase I footprinting or EMSA analysis, respectively. Although putative AP-1-binding sites at −902, −856, and −339 are in the regions found to have increased protein binding after TBH treatment on DNase I footprinting analysis, no change in AP-1 binding activity was detected with EMSA. TBH treatment also led to increased Sp1 binding to the Sp1 site at −689 to −680, which is in the region detected to have increased protein binding on DNase I footprinting analysis but devoid of AP-1 site.

To confirm that AP-1 binding led to transactivation of the gene, cells were infected with dominant-negative c-*jun* prior to TBH treatment. Blocking AP-1 activity led to a fall in the basal mRNA level of all three genes and their constitutive promoter activities. This finding is similar to what has been reported also for the human GCL subunit promoters and GCLC mRNA level, where AP-1 activity is required for constitutive activity and basal expression (27–29). However, blocking AP-1 activity also blocked significantly the TBH-mediated increase in the promoter activity of the rat GCL subunits and GS as well as the mRNA levels of all three genes. The inhibition was not complete, however, which may reflect that fact that increased AP-1 binding was not completely abolished (see Fig. 7). Of interest is that blocking AP-1 activity also blocked the increase in Sp1 binding (Fig. 8). Recently direct physical interaction between c-*jun* and Sp1 has been demonstrated, and c-*jun* was able to synergize the transactivation of Sp1 without actually binding to the DNA itself (30, 31). Consistent with this, we also did not detect c-*jun* binding to the Sp1 site (Fig. 8). Our data suggest that dominant-negative c-*jun* interacted with Sp1 and prevented its binding to the GS promoter. This can explain the decrease in Sp1 binding activity under basal conditions as well as in response to TBH treatment (Fig. 8). It is likely that functional cooperation between c-*jun* and Sp1 also contributes to the TBH-mediated induction of GS. Finally, our data do not preclude other AP-1-independent mechanism(s) in contributing to the TBH-induced up-regulation of the GSH synthetic enzymes.

In summary, we have cloned and analyzed the 5′-flanking region of the rat GS gene. The rat GS promoter contains both positive and negative regulatory regions. NF1 is an important repressor of the GS promoter. By using the cloned rat GCL and GS promoters, we have identified AP-1 activity as essential for their constitutive expression and for the up-regulation of all three genes in response to TBH.

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